Reactivity of a functional carbonyl moiety in bovine aortic lysyl oxidase

Evidence against pyridoxal 5'-phosphate

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Previous studies have pointed towards a cofactor role for pyridoxal 5'-phosphate (PLP) in lysyl oxidase, the enzyme that generates the peptidyl aldehyde precursor to the lysine-derived cross-linkages in elastin and collagen. The nature of a carbonyl moiety in purified bovine aortic lysyl oxidase was explored in the present study. A PLP dinitrophenylhydrazone could not be isolated from lysyl oxidase, although corresponding preparations of aspartate aminotransferase, a PLP-dependent enzyme, yielded this derivative, as revealed by h.p.l.c. Analysis of lysyl oxidase for PLP after reduction of the enzyme by $NaBH_4$, a procedure that converts PLP–protein aldimines into stable 5'-phosphopyridoxyl functions, also proved negative in tests using monoclonal antibody specific for this epitope. Lysyl oxidase was competitively inhibited by phenylhydrazine, and inhibition became irreversible with time at 37 °C, displaying a first-order inactivation rate constant of 0.4 min^{-1} and K_I of 1 μM. [^{14}C]Phenylhydrazine was covalently incorporated into the enzyme in a manner that was prevented by prior modification of the enzyme with β-aminopropionitrile, a specific active-site inhibitor, and which correlated with functional active-site content. The chemical stability of the enzyme-bound phenylhydrazine exceeded that expected of linkages between PLP and proteins. The absorption spectrum of the phenylhydrazine derivative of lysyl oxidase was clearly distinct from that of the phenylhydrazone of PLP. It is concluded that lysyl oxidase contains a carbonyl cofactor that is not identical with PLP and that is bound to the enzyme by a stable chemical bond.

INTRODUCTION

Lysyl oxidase is a copper-dependent amine oxidase that initiates covalent cross-linkage formation within the polypeptide chains of elastin and collagen by oxidatively deaminating certain lysine residues in these proteins to peptidyl α-aminoadipic δ-semialdehyde. Spontaneous condensation of the aldehyde with other lysine or aldehyde residues can form inter- and intra-chain cross-linkages, which stabilize and insolubilize these connective-tissue proteins (Pinnell & Martin, 1968; Siegel, 1979). Bovine aortic lysyl oxidase also oxidizes simple primary amines and diamines (Trackman & Kagan, 1979) as well as various lysine-containing peptides and basic proteins (Kagan et al., 1980, 1983, 1984).

The presence of a carbonyl-containing prosthetic group in lysyl oxidase has long been suspected. Severe connective-tissue defects follow upon the treatment of chick embryos with ureides, hydrazines or hydrazides, each of which is reactive towards carbonyl functions (Levene, 1961), and the administration of iproniazid altered normal cross-linkage patterns in chick elastin and collagen (Carrington et al., 1984). Further, lysyl oxidase is inhibited in vitro by micromolar to millimolar concentrations of phenylhydrazine (Harris et al., 1974; Kagan et al., 1974), isoniazid (Kagan et al., 1974; Arem & Misiorowski, 1976) and hydroxylamine (Kagan et al.,

1974), consistent with the presence of a functional carbonyl moiety in the enzyme. Various nutritional studies have documented that vitamin B-6-deficient diets result in decreased or altered patterns of cross-linkages in connective-tissue proteins (Starcher, 1969; Fuji et al., 1979; Myers et al., 1985), suggesting that PLP may function in this role in lysyl oxidase. This possibility was also supported by the observation that [G-^{3}H]pyridoxine injected into 16-day chick embryos co-chromatographed with lysyl oxidase activity extracted from these chicks (Murray et al., 1978). Moreover, chick cartilage lysyl oxidase activity decreased in pyridoxine-deficient chicks, although enzyme activity was not as affected in aorta (Murray & Levene, 1977). The potential complexity of these studies in vitro was indicated by the apparently contrary observation that cross-linked collagen appeared to be increased in vitamin B-6-deficient rats (Tane et al., 1976), inconsistent with a decrease in lysyl oxidase activity. More direct support for a role for PLP was provided by the finding that the loss of activity accompanying dialysis of purified chick aortic lysyl oxidase was largely restored by addition of PLP to the dialysed enzyme, and that fluorescence emission spectra of the cyanide or semicarbazide adducts of this enzyme resembled those of the corresponding derivatives of authentic PLP (Bird & Levene, 1982). Nevertheless, differences were noted between the cyanide spectra of the enzyme and model compound, and it was calculated from

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spectral analysis that this enzyme contained 2.26 mol of PLP/mol of enzyme subunit, a value seemingly difficult to reconcile with a mechanistic role for this carbonyl moiety (Bird & Levene, 1982). In the present study we have undertaken the characterization of a reactive carbonyl function in purified bovine aorta lysyl oxidase by immunological, chemical and spectral means. Kinetic and binding studies of the reactivity of phenylhydrazine with this enzyme are consistent with the localization of carbonyl reactivity at the active site, although the spectral, immunological and chemical studies argue against the presence of PLP in this enzyme.

MATERIALS AND METHODS

Materials

[U-^{14}C]Phenylhydrazine (7.5 mCi/mmol) was obtained from ICN Pharmaceuticals, Irvine, CA, U.S.A. n-Butylamine and phenylhydrazine hydrochloride were purchased from Fisher Scientific Co., Fair Lawn, NJ, U.S.A. 2-Amino[3-^{14}C]propionitrile hydrochloride (5 mCi/mmol) was prepared from Na^{14}CN and 2-bromoethylamine as described previously (Tang et al., 1983). 2,4-Dinitrophenylhydrazine, 2-aminopropionitrile fumarate, pyridoxal hydrochloride, PLP, aspartate aminotransferase (2000 units/mg) and phosphorylase b (30 units/mg) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Formula 963 scintillation fluid was obtained from New England Nuclear, Boston, MA, U.S.A. F(ab')$_2$ fragment of goat anti-(mouse IgG) [F(ab')$_2$-fragment-specific] second antibody was from Cappel Laboratories, Malvern, PA, U.S.A. F(ab')$_2$ fragment of mouse anti-peroxidase complexed with peroxidase was purchased from Pel-Freez Biologicals, Rogers, AR, U.S.A.

Enzyme assays

Lysyl oxidase activity was assayed against an insoluble elastin substrate prepared from chick-embryo aortas pulsed in organ culture with L-[4,5-^{3}H]lysine as described by Pinnell & Martin (1968). Enzyme assay mixtures included 184000 d.p.m. of the elastin substrate in 0.15 M-NaCl/0.1 M-sodium borate buffer, pH 8.0, in a total volume of 750 μl, and were incubated for 2 h at 37 °C. Tritiated water formed during the incubation was collected by vacuum distillation and quantified by liquid-scintillation spectrometry of 0.5 ml portions of the distillates. All activity data were corrected for enzyme-free controls. One enzyme unit was defined as 1 d.p.m. of ^{3}H released by enzyme action in 2 h per 0.5 ml portion of distillate.

Lysyl oxidase was also assayed against n-butylamine as substrate by a peroxidase-coupled fluorescence method (Trackman & Kagan, 1979). Reaction mixtures contained n-butylamine at the concentrations indicated, 40 μg of horseradish peroxidase, 0.7 mM-homovanillic acid, 1.2 M-urea and 16 mM-potassium phosphate buffer, pH 8.0, in a total volume of 2 ml. Approx. 1400 units of lysyl oxidase were added to initiate oxidation. Fluorescence was continuously monitored at 55 °C at an excitation wavelength of 315 nm and an emission wavelength of 425 nm. Enzyme-dependent production of H$_2$O$_2$ was quantified by reference to standard plots relating mol of H$_2$O$_2$ to fluorescence units.

Enzyme purification

Lysyl oxidase was purified from bovine aorta by a modification (Williams & Kagan, 1985) of the method previously described (Kagan et al., 1979). This modified procedure substitutes Cibacron Blue–Sepharose for the DEAE-cellulose chromatographic step and thus does not separate the ionic variants of lysyl oxidase from each other. Enzyme protein was assayed by the procedure of Lowry et al. (1951), with bovine serum albumin as the reference standard. Specific activities of the purified enzyme preparations used in this study ranged from 400000 to 1400000 units/mg, in assays against the tritiated elastin substrate. Studies on the co-purified mixture of these variants appear to be justified by prior findings that the individual variants each have an M_r of approx. 32000 in SDS/polyacrylamide-gel electrophoresis and that the substrate specificity and inhibitor profiles as well as the peptide maps of partial proteinase digests of each are quite similar or identical (Sullivan & Kagan, 1982). Thus it is quite likely that each variant has the same mechanism of action and that the primary and higher-ordered structures are at least extremely similar.

Determination of the 2,4-dinitrophenylhydrazone of PLP

Dialysis bags were soaked in 0.1 M-EDTA at 70 °C, and then repeatedly washed in distilled water at 70 °C to remove hydrazine-reactive contaminants. Aspartate aminotransferase (0.23 mg; 2000 units/mg) and lysyl oxidase (0.57 mg; 1000000 units/mg) were dialysed against distilled water, adjusted to pH 2 with HCl, and then heated at 65 °C for 30 min. After evaporation to dryness each enzyme residue was extracted three times with 50 μl of aq. 50% (v/v) methanol, the extracts were pooled and centrifuged, and the supernatants were decanted and evaporated to dryness under N$_2$. Each residue was redissolved in 50 μl of distilled water, mixed with 6.1 nmol of 2,4-dinitrophenylhydrazine and 5 μl of 12 M-HCl and then refluxed at 90 °C for 5 min. The reaction mixtures were evaporated under N$_2$, redissolved, and clarified by centrifugation, and the supernatants were analysed by h.p.l.c., with an Altex 5 μm-particle-size reversed-phase Ultrasphere-ODS column and elution with an isocratic mobile phase of 57.5% methanol/42.4% water/0.1% triethylamine, pH 7.0, at a flow rate of 1 ml/min. The eluate was monitored for absorbance at 405 nm.

Modification with [^{14}C]phenylhydrazine

Lysyl oxidase (of various specific activities) or 25 μg of bovine serum albumin, lysozyme or carbonic anhydrase each was incubated with 1.0 μM-[U-^{14}C]phenylhydrazine at 37 °C for 1.5 h in 1 ml of 16 mM-potassium phosphate buffer, pH 8.0. Protein-bound reagent was quantified by liquid-scintillation spectrometry after extensive dialysis of the modified proteins against 16 mM-potassium phosphate buffer, pH 8.0. In some experiments lysyl oxidase was pretreated with 0.1 M-NaBH$_4$ for 1 h at 25 °C, or with 1.0 μM-hydrazine for 1.5 h at 37 °C, pH 8.0, or with 10 μM-2-aminopropionitrile for 2 h in 16 mM-potassium phosphate buffer, pH 7.8, as described by Tang et al. (1983), and then extensively dialysed against 16 mM-potassium phosphate buffer, pH 8.0, before incubation with [U-^{14}C]phenylhydrazine.

SDS/polyacrylamide-gel electrophoresis was performed in 12.5% cross-linked polyacrylamide gel slabs by

the method of Laemmli (1970). Protein samples were dialysed against distilled water and freeze-dried in preparation for electrophoresis. Gels were stained with Coomassie Blue, destained and dried. The dried gel was next exposed to Kodak XAR-5 X-ray film for a period of 2 days and then developed.

Immunological assay for PLP

Analyses for borohydride-reducible PLP–enzyme aldimines were carried out by a modification of the procedure of Viceps-Madore *et al.* (1983) that relies upon the detection of protein-bound PLP by immunoblot analysis of borohydride-reduced proteins. Protein-bound PLP aldimine-linked to ε-amino functions of lysine residues are thus reduced to stable 5'-phosphopyridoxyl-lysine moieties, which are then identified by monoclonal antibody specific for this epitope after SDS/ polyacrylamide-gel electrophoresis and transfer of the reduced protein to nitrocellulose paper. Lysyl oxidase (75 μg; 800000 units/mg) was reduced in 4 ml of 0.1 M-NaBH$_4$ in 6 M-urea/16 mM-potassium phosphate buffer, pH 7.7, for 1 h at 25 °C. An equivalent amount of lysyl oxidase was reduced under the same conditions in the presence of 5 mM-PLP. Phosphorylase *b* was reduced under the same conditions but without added PLP. The reduced proteins were exhaustively dialysed against distilled water and freeze-dried. The proteins were subjected to SDS/polyacrylamide-gel electrophoresis on slabs of 7.5% cross-linked polyacrylamide, and the electrophoretograms were horizontally electro-eluted on to nitrocellulose paper in accordance with Towbin *et al.* (1980). The nitrocellulose sheet was then incubated in a solution of 1% (w/v) bovine serum albumin and 2.5% (v/v) PLP-depleted human plasma in phosphate-buffered saline (0.15 M-NaCl/10 mM-sodium phosphate buffer, pH 7.4) for 30 min at 37 °C. This solution was decanted, and the nitrocellulose was incubated in 0.3% H$_2$O$_2$ in phosphate-buffered saline for 30 min and then washed three times in phosphate-buffered saline. Immune ascites fluid obtained from mice inoculated with the E6(2)2 hybridoma (Viceps-Madore *et al.*, 1983) was diluted 1:500000 and incubated with the blot. The blot was washed in phosphate-buffered saline containing 0.05% Tween 80 and then incubated with a 1:10000 dilution of F(ab')$_2$ fragment of goat anti-(mouse IgG) [F(ab')$_2$-fragment-specific] second antibody. After a washing with phosphate-buffered saline containing 0.05% Tween 80, the blot was incubated with a 1:10000 dilution of F(ab')$_2$ fragment of mouse anti-peroxidase complexed with peroxidase. After a washing, the blot was developed with a solution of diaminobenzidine (0.3 mg/ml) and 1.7 mM-H$_2$O$_2$ in 50 mM-Tris/HCl buffer, pH 7.6, at 25 °C until the desired staining intensity was obtained.

RESULTS

H.p.l.c. analysis of dinitrophenylhydrazones

In view of previous evidence indicating the presence of PLP in chick aortic lysyl oxidase (Bird & Levene, 1982), initial efforts were directed at detecting such a cofactor in lysyl oxidase. The procedure is based on the previously established lability of the PLP–enzyme aldimine bond in aminotransferases to acidic or basic conditions (Jenkins & Sizer, 1960), as well as the known reactivity of PLP
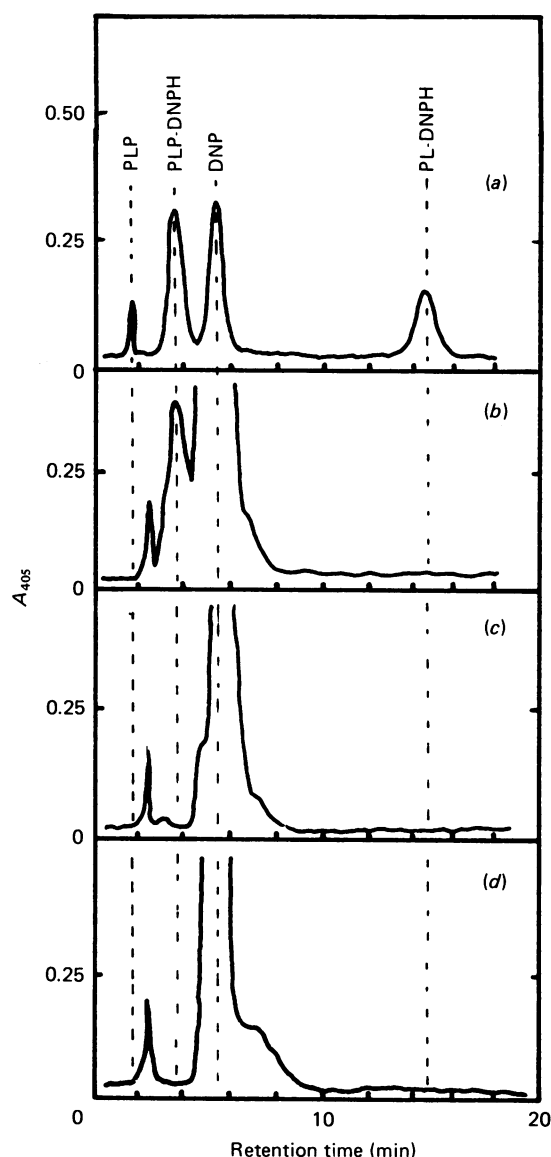

Fig. 1. Resolution of products of reaction with dinitrophenylhydrazine by h.p.l.c.

(*a*) Standards: 2 nmol of PLP (PLP); 1 nmol of PLP dinitrophenylhydrazone (PLP-DNPH); 2 nmol of dinitrophenylhydrazine (DNPH); 1 nmol of pyridoxal dinitrophenylhydrazone (PL-DNPH). (*b*) Profile of reaction products of aspartate aminotransferase (2.4 nmol of enzyme, 4.9 nmol of PLP equivalents). (*c*) Negative control: distilled water carried through same conditions as in (*b*). (*d*) Profile of reaction products of lysyl oxidase (0.57 mg; 1000000 units/mg) treated identically as in (*b*).

toward arylhydrazines (Shimomura *et al.*, 1978). After solubilization and reaction of carbonyl prosthetic groups, the phenylhydrazone is isolated and analysed by h.p.l.c., as detailed in the Materials and methods section. As shown (Fig. 1*a*), the dinitrophenylhydrazone prepared from authentic PLP is eluted at 3.5–3.75 min, well separated from excess dinitrophenylhydrazine, PLP or the dinitrophenylhydrazone of pyridoxal. As shown in Fig. 1(*b*), the product isolated from a sample of chemically modified aspartate aminotransferase yields a peak emerging at the position expected for the dinitrophenyl-

hydrazone of PLP. The yield of this product was estimated to be 2.45 nmol or 50% of the theoretical value of 4.9 nmol of PLP present in the quantity of enzyme used for this analysis. The product appearing on h.p.l.c. was quantified by comparison of the integrated area of the peak with those of known quantities of the PLP phenylhydrazone standard. The profile of the h.p.l.c. eluate of the corresponding preparation of a control sample that lacked PLP or any potential protein sources of PLP (Fig. 1c) did not contain a peak corresponding to PLP dinitrophenylhydrazone, as expected. Similarly, h.p.l.c. of the corresponding preparation of lysyl oxidase also lacked a peak corresponding to the dinitrophenyl-hydrazone of PLP (Fig. 1d), although this profile represents an analysis of 17.8 nmol (0.57 mg) of lysyl oxidase of specific activity 1000000 units/mg. The peak eluted at 2.5–2.9 min found in the lysyl oxidase profile (Fig. 1d) was also found in the negative control (Fig. 1c) and may represent a reactive contaminant of the dialysis tubing, since identical treatment and analyses of control solutions that were not in contact with dialysis membranes did not display this peak. Previous studies have indicated that preparations of purified lysyl oxidase contain both active and inactive enzyme molecules (Tang et al., 1983, 1984). The results obtained by Tang et al. (1983, 1984) indicate, however, that the functional active-site content can be assessed from the molar incorporation of 2-amino[^{14}C]propionitrile into lysyl oxidase. Thus 2-aminopropionitrile acts as a mechanism-based irreversible enzyme inhibitor that is not turned over by lysyl oxidase and hence acts as an active-site titrant. Calculations based on this assumption and using the data of Tang et al. (1983) predict that completely active enzyme has a specific activity of 4000000 units/mg, and on this basis the amount of enzyme units (570000) analysed in the study shown in Fig. 1(b) approximates to 4.6 nmol of functional active sites. Since the present technique appears more than sufficiently sensitive to detect this amount of PLP, this result argues against the presence of non-covalently bound or aldimine-linked PLP in bovine aortic lysyl oxidase.

Reactivity of lysyl oxidase with phenylhydrazine

Although previous studies have noted the inhibition of lysyl oxidase by hydrazine and aromatic hydrazines (Harris et al., 1974; Kagan et al., 1974), the nature of the inhibition has not been sufficiently characterized to conclude that such modification is specific for the active site. Further, although a dinitrophenylhydrazone was not solubilized from lysyl oxidase in the present study (Fig. 1), it remained possible that a carbonyl moiety may be linked to the enzyme by a bond not displaceable by the present method but which may form a phenylhydrazone that remains covalently linked to the enzyme. Purified bovine aortic lysyl oxidase was therefore modified with phenylhydrazine, and the kinetics of inhibition and degree of covalent complex formation were assessed. Phenylhydrazine rather than dinitrophenylhydrazine was selected for this purpose, since the former is available as a ^{14}C-labelled compound.

Phenylhydrazine competitively inhibits lysyl oxidase activity against n-butylamine, as shown by the intersection of the lines at the $1/v$ axis in the double-reciprocal Lineweaver & Burk (1934) plot of the assay data (Fig. 2). The K_I for phenylhydrazine was calculated from these data to be 1.2 μM. The reversibility of the inhibition was
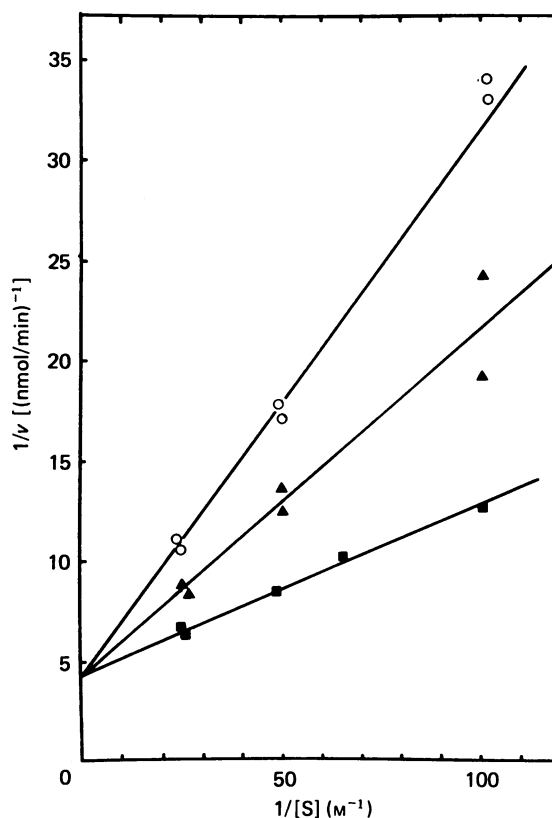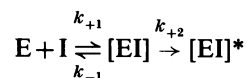

Fig. 2. Double-reciprocal plot of the inhibition by phenylhydrazine of lysyl oxidase activity against n-butylamine

Assays were in the absence (■) or in the presence of 1.96 μM-(▲) or 3.24 μM- (○) phenylhydrazine. For experimental details see the text.

assessed by incubating enzyme with phenylhydrazine at 37 °C for various periods of time and then diluting samples of the preincubated enzyme into the peroxidase-coupled assay. This experiment revealed that phenyl-hydrazine caused a time-dependent loss in enzyme activity. Extensive dialysis of samples of the preincubated mixtures did not restore enzyme activity, confirming the irreversible nature of the inhibition by phenylhydrazine. A plot of the logarithm of the residual activity against the time of preincubation yields a linear relationship (Fig. 3a) consistent with a first-order process and suggesting that the event that results in enzyme inactivation does not reflect a random bimolecular collision between phenyl-hydrazine and the susceptible enzyme residue but rather proceeds from an EI complex in which the inhibitor is reversibly bound, according to:

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} [EI] \overset{k_{+2}}{\rightarrow} [EI]^*$$

where [EI]* is the inactivated EI complex, $k_{-1}/k_{+1} = K_I$, and k_{+2} is the limiting first-order rate constant for inactivation (Walsh, 1979). A secondary double-reciprocal plot of the $k_{inact.}$ values versus the concentration of phenylhydrazine yields a straight line consistent with a site-saturation phenomenon and the intercept of which at the ordinate is equal to the reciprocal of k_2 (Walsh, 1979), in this case yielding a k_{+2} value of 0.4 min^{-1} (Fig. 3b). Consistent with the competitive nature of the inhibition
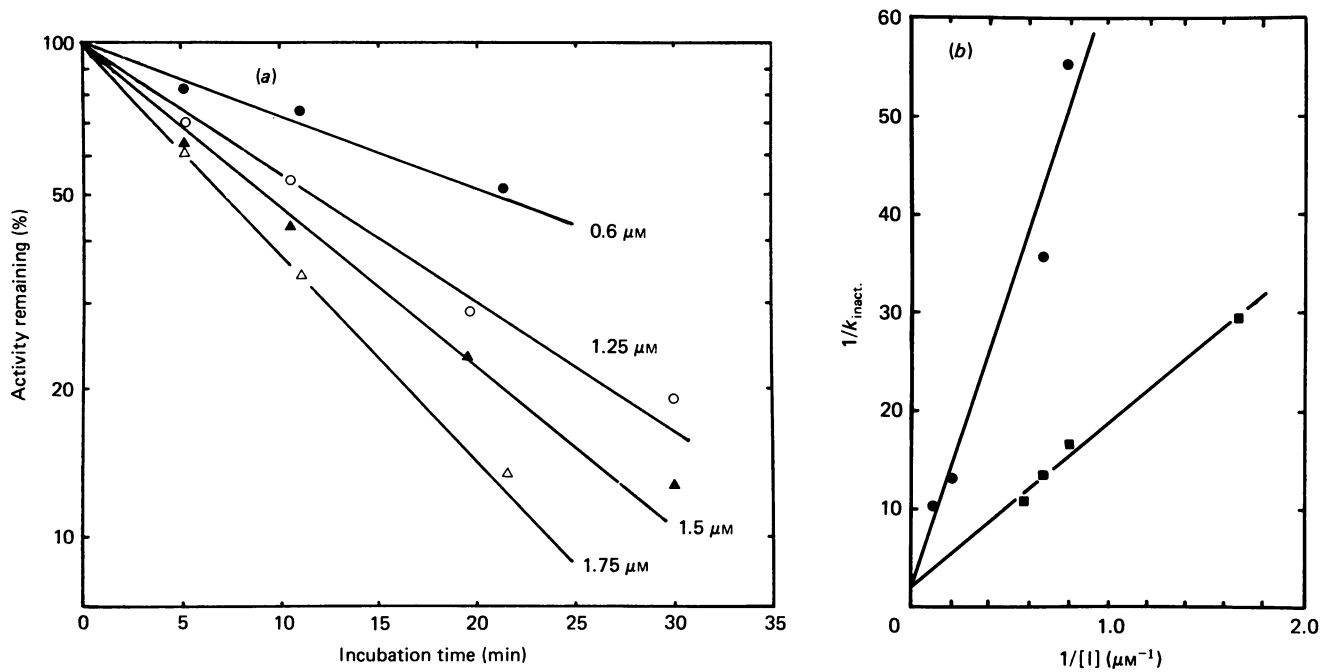
Fig. 3. Time-dependent irreversible inhibition of lysyl oxidase by phenylhydrazine

(a) Lysyl oxidase (45000 units/ml) was preincubated at 37 °C in 16 mM-potassium phosphate buffer, pH 8.0, in the presence of the indicated concentrations of phenylhydrazine, and residual activity was assayed by dilution of portions into the fluorimetric coupled assay system. Enzyme preincubated in the absence of phenylhydrazine retained its full activity over the time span of the experiment. (b) $k_{inact.}$ values obtained in the presence (●) and in the absence (■) of 20 mM-n-butylamine.
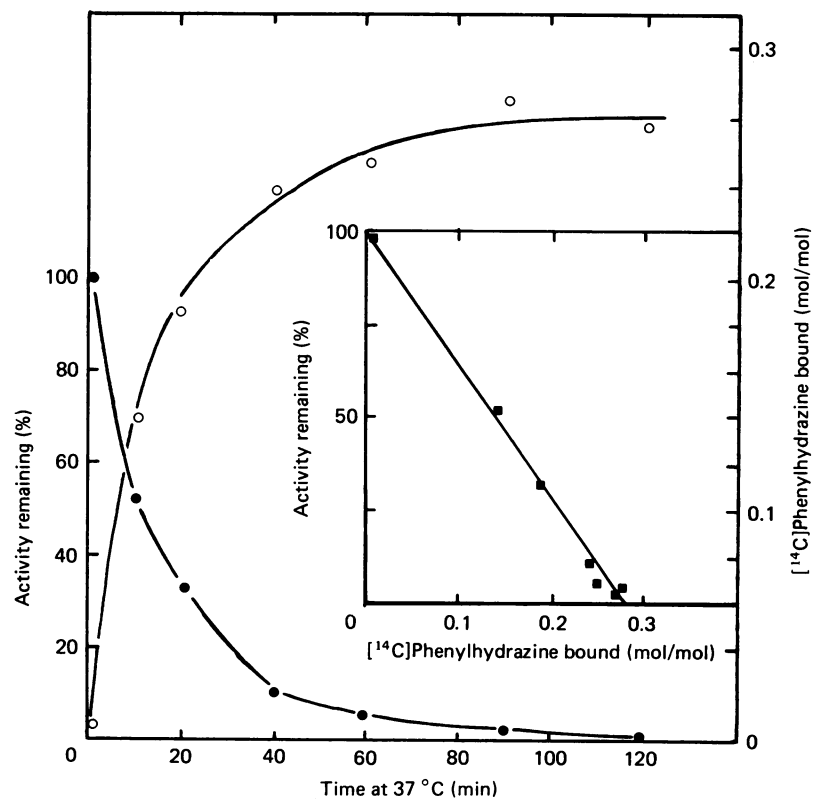
Fig. 4. Correlation of time-dependent covalent incorporation of [¹⁴C]phenylhydrazine (○) with enzyme inactivation (●)

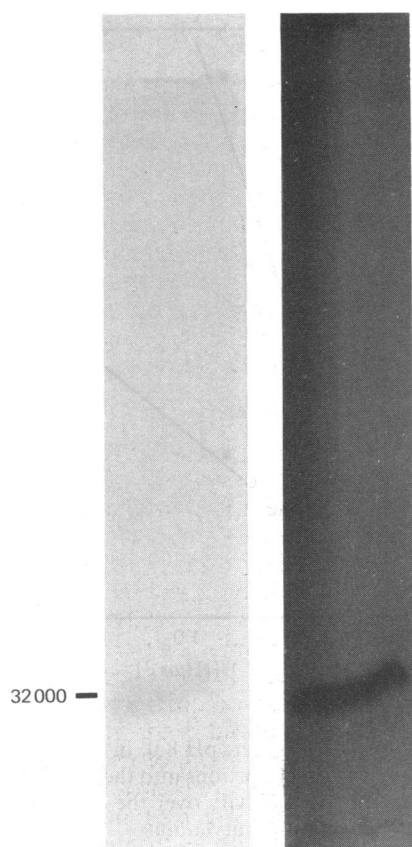Lysyl oxidase was modified with 1.0 μM-[¹⁴C]phenylhydrazine as described in the Materials and methods section.

Fig. 5. SDS/polyacrylamide-gel electrophoresis of lysyl oxidase modified with [U-^{14}C]phenylhydrazine

Left-hand lane, enzyme (M_r 32000) stained with Coomassie Blue; right-hand lane, autoradiogram of the same gel.
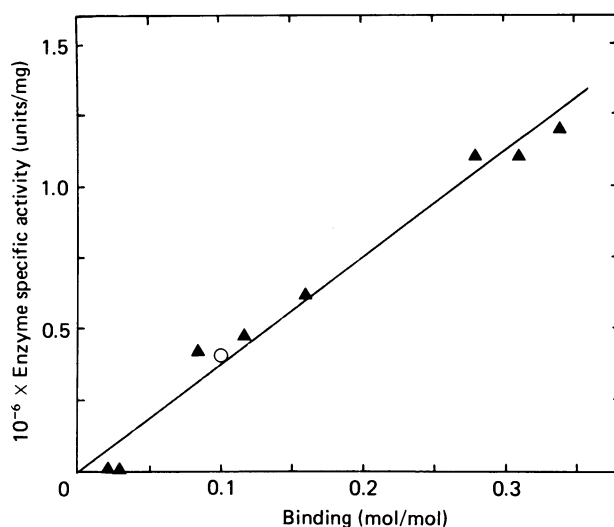

Fig. 6. Correlation of radioactive-ligand binding with the specific activity of lysyl oxidase

Lysyl oxidase preparations of various specific activities were treated with 1 μM-[^{14}C]phenylhydrazine for 1.5 h at 37 °C in 16 mM-potassium phosphate buffer, pH 8.0, followed by extensive dialysis. The results are represented by ▲ symbols, the two points on the abscissa representing enzyme samples treated with [^{14}C]phenylhydrazine after complete inactivation of the enzyme by 10 μM-2-aminopropionitrile. The ○ symbol (datum of Tang et al., 1983) refers to enzyme modified only with 2-amino [^{14}C]propionitrile.

shown in Fig. 2, the rate of development of irreversible inactivation is markedly lowered by the presence of 20 mM-n-butylamine in the preincubation mixture of enzyme and phenylhydrazine (Fig. 3b).

Lysyl oxidase was incubated with 1.0 μM-[^{14}C]-phenylhydrazine for 1.5 h as described above and exhaustively dialysed to assess the possibility that a phenylhydrazone forms that does not dissociate from the enzyme. Enzyme treated in this fashion retained 0.28 mol of phenylhydrazine/mol of protein and was completely inactivated. Incorporation of radioactivity was decreased to 0.02–0.03 mol/mol of enzyme if the enzyme was first irreversibly inhibited by preincubation with 2-amino-proprionitrile or if the enzyme was first reduced with NaBH$_4$ or inactivated with hydrazine, the last two reagents exhibiting selectivity for carbonyl functions. Carbonic anhydrase, lysozyme and bovine serum albumin incorporated similar negligible amounts of ^{14}C (0.01–0.03 ml/mol) when modified under the same conditions as lysyl oxidase. The time-dependent incorporation of label into lysyl oxidase exhibits a reciprocal relationship with remaining enzyme activity (Fig. 4), with the complete inactivation of enzyme occurring upon the incorporation of 0.28 mol of reagent/mol of enzyme (Fig. 4 inset). These results are consistent with the specific modification of a carbonyl residue that is functional in catalysis.

The [^{14}C]phenylhydrazine–lysyl oxidase complex ex-

hibited significant chemical stability. Thus radioactivity bound to lysyl oxidase was decreased only by 14% upon incubation of the complex at 55 °C for 1 h in 0.1 M-HCl or by 24% when incubated in 1 M-HCl under these conditions. None of the protein-bound radioactivity was displaced upon incubation in 1 M-NaOH under these conditions. Moreover, the radioactivity of the labelled enzyme complex was stable to displacement by boiling in 2% (w/v) SDS for 5 min in the presence of 2-mercaptoethanol, since 92% of the applied radioactivity migrated upon SDS/polyacrylamide-gel electrophoresis to a position equivalent to an M_r value of 32000, which is the M_r of lysyl oxidase under these conditions (Fig. 5). Thus enzyme-bound phenylhydrazine appears to be covalently linked to an active-site carbonyl moiety that in turn appears to be covalently linked to the enzyme protein.

Although the stoichiometry of incorporation of radioactive phenylhydrazine into the native enzyme is less than unity, the degree of incorporation correlates with the specific activity of the various preparations of purified lysyl oxidase modified with this radioactive compound in these studies (Fig. 6). Moreover, the molar incorporation of 2-amino[1,2-^{14}C]propionitrile into a sample of native lysyl oxidase (Tang et al., 1983) correlates with the linear relationship described by the incorporation of [^{14}C]phenylhydrazine, as also shown in Fig. 6. These results thus support the conclusion that each reagent titrates functional active-site content, as previously inferred by the kinetic and labelling characteristics of 2-aminopropionitrile with lysyl oxidase (Tang et al., 1983). This linear relationship also predicts that fully
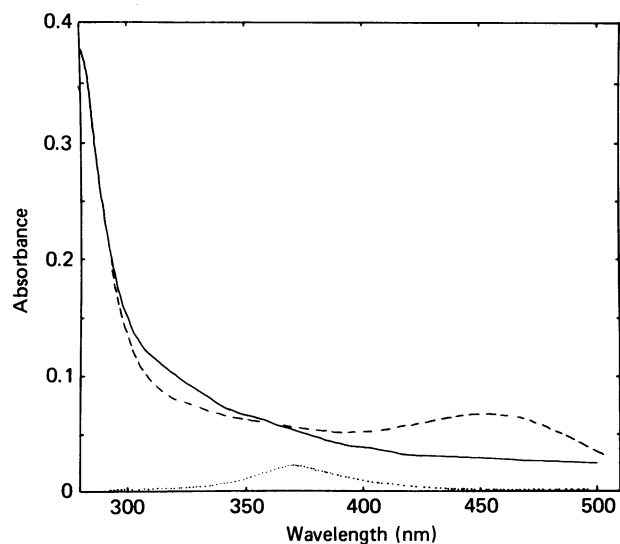
Fig. 7. Absorption spectra

Equivalent amounts of lysyl oxidase (0.2 mg; 1020000 units/mg; 1.6 nmol of active sites) were dialysed against distilled water with (— — —) or without (———) prior treatment with [U-¹⁴C]phenylhydrazine (1.0 μM in 16 mM-potassium phosphate buffer, pH 8.0, at 37 °C for 1.5 h); · · · · ·, 1.6 nmol of PLP phenylhydrazone or pyridoxal phenylhydrazone. All spectra were of samples in 1 ml of 8 M-urea/16 mM-potassium phosphate buffer, pH 7.7.

active enzyme would have a specific activity of 4000000 units/mg of enzyme protein, assuming that fully active enzyme would incorporate 1 mol of 2-aminopropionitrile or of phenylhydrazine per mol.

Phenylhydrazone absorption spectra

The absorption spectra of the phenylhydrazine derivatives of lysyl oxidase and PLP are compared in Fig. 7. The modified form of lysyl oxidase exhibits an abosrption maximum at 455 nm in 8 M-urea, whereas that of authentic PLP phenylhydrazone occurs at 375 nm in this solvent. The spectrum of phenylhydrazine-treated lysyl oxidase was not significantly altered by digestion of the modified protein with trypsin, suggesting that the spectral features of the intact protein are largely independent of interactions of the chromophore with the intact protein in 8 M-urea, as would be expected.

Immunological analysis for PLP

Lysyl oxidase was also analysed by an immunological procedure specific for protein-bound PLP. This procedure assumes that PLP is aldimine-linked to a lysine side chain of the apoprotein and utilizes monoclonal antibody specific for protein-bound PLP that has been reduced by $NaBH_4$ to the phosphopyridoxyl-lysine secondary amine derivative (Viceps-Madore et al., 1983), as described in the Materials and methods section. The SDS/polyacrylamide-slab-gel electrophoretogram of protein standards, 10 μg of lysyl oxidase (equivalent to 37.5 pmol of functional active sites) reduced in the presence and in the absence of 5 mM-PLP and 10 μg of phosphorylase b reduced in the absence of PLP are shown in Fig. 8(a) in lanes 1, 2, 3 and 4 respectively. Fig. 8(b) shows a Western immunoblot of a similar gel with additional lanes 5 and 6 containing 1 μg and 0.1 μg of reduced phosphorylase b respectively. The lysyl oxidase sample reduced in the presence of PLP is strongly positive towards the anti-phosphopyridoxyl-lysine antibody (lane 3 in Fig. 8b) whereas the lysyl oxidase sample reduced in the absence of added PLP (lane 2 in Fig. 8b) appeared completely
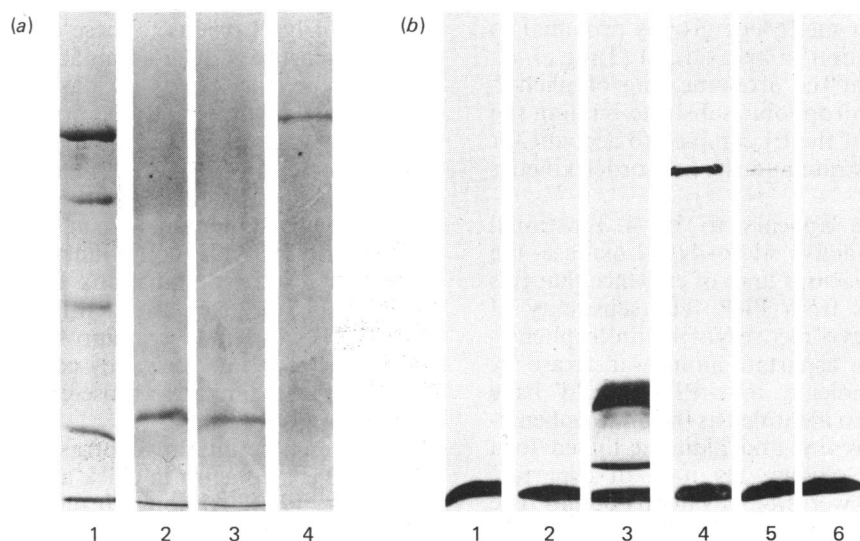
Fig. 8. Immunoblot analysis for PLP in proteins

(a) Coomassie Blue-stained SDS/polyacrylamide-gel electrophoretogram. Lane 1, M_r markers; lane 2, 10 μg of reduced lysyl oxidase; lane 3, 10 μg of lysyl oxidase reduced in the presence of 5 mM-PLP; lane 4, 10 μg of reduced phosphorylase b. (b) Western immunoblot analysis of electrophoresed proteins with monoclonal antibody specific for 5′-phosphopyridoxyl epitope. Lanes 1-4 contain samples as identified in the Coomassie Blue-stained gel on the left. Lanes 5 and 6 contain 1.0 μg and 0.1 μg of reduced phosphorylase b respectively.

negative. The limit of detection of this technique is illustrated by the positive response to 0.1 μg of phosphorylase b in lane 6 in Fig. 8(b), which contains 1.1 pmol of PLP. In contrast, the negative reaction obtained with 10 μg of reduced lysyl oxidase in lane 2 in Fig. 8(b) represents 330 pmol of enzyme or 37.5 pmol of functional active site. The positive reaction with lysyl oxidase reduced in the presence of PLP provides evidence that lysyl oxidase, itself, does not inhibit the antibody-dependent reaction.

DISCUSSION

The mechanism of action of lysyl oxidase is not yet understood. This circumstance is largely due to the uncertainty that still exists about the chemical identity of a carbonyl moiety which previous studies have implicated as a cofactor. In part, the present results permit the conclusion that the carbonyl moiety of the purified bovine aortic enzyme is an active-site function and is probably involved in the catalytic mechanism. This conclusion is drawn from the irreversible loss of activity upon modification of the enzyme with phenylhydrazine in a manner that is competitively protected against by the presence of the lysyl oxidase substrate n-butylamine, and by the prevention of [^{14}C]phenylhydrazine incorporation into the enzyme by prior covalent modification of the active site by 2-aminopropionitrile. The first-order irreversible loss in enzyme activity suggests that the events that lead to inactivation initially involve the binding of phenylhydrazine specifically at the active site to form a reversible EI complex. Covalent modification and thus inactivation of the enzyme proceeds from this complex as if it were a unimolecular species and at a velocity that is sufficiently rate-limiting to be measureable under the specified conditions. Such kinetic behaviour is characteristic of enzyme affinity labels that are recognized by the substrate-binding site and that carry reactive functional groups. Since lysyl oxidase is able to utilize benzylamine, an aromatic amine, as a substrate (Trackman, 1980) and otherwise prefers apolar methylene groups proximal to the primary amine function to be oxidized (Tang et al., 1984), it is possible that the aromatic ring of phenyl-hydrazine occupies a hydrophobic substrate-binding site upon initial formation of the EI complex to account for the competition by substrate and the first-order kinetics obtained.

Although there thus appears to be a functional carbonyl moiety at the active site of lysysl oxidase, the present study provides various lines of evidence that this carbonyl moiety differs from PLP. The sensitivity of detection and the efficiency of recovery of the dinitrophenyl-hydrazone of PLP from aspartate aminotransferase by the h.p.l.c. method indicate that PLP would have similarly reacted and been identified as the dinitrophenyl-hydrazone if it were present and aldimine-linked to a lysine residue in lysyl oxidase, as it is in aspartate aminotransferase, or if it were non-covalently bound. The limits of detection of the immunological assay are clearly more than sufficient to have detected reduced 5'-phosphopyridoxyl-lysine in lysyl oxidase, as well, even accounting for the partial content of functional active sites. The completely negative results with both procedures as well as the quantitative and qualitative differences noted between the spectra of the covalent phenylhydrazine adduct of lysyl oxidase and PLP phenylhydrazone argue

against the conclusion that lysyl oxidase contains non-covalently bound or aldimine-linked PLP.

The stability of the [^{14}C]phenylhydrazine–lysyl oxidase adduct to acid and base as well as to heat denaturation of the protein indicates that the carbonyl moiety is linked to the protein by a reasonably stable chemical bond. Although it is possible that a PLP cofactor might participate in an ester or anhydride linkage with the enzyme through its phenolic hydroxy or phosphoryl moieties, such linkages also would not be expected to exhibit such chemical stability, nor does there appear to be prior evidence for such PLP–protein linkages. We have also noted (P. R. Williamson, J. M. Kittler, J. W. Thanassi & H. M. Kagan, unpublished work) that the partial loss of activity accompanying dialysis of the bovine aortic enzyme is not restored by addition of Cu^{2+} or PLP separately or in combination to the dialysed enzyme, thus contrasting with the apparent restoration of activity by addition of PLP to the dialysed chick aortic enzyme (Bird & Levene, 1982). In summary, it appears very unlikely that PLP is present in bovine aortic lysyl oxidase.

Although the chemical identity of the carbonyl function in lysyl oxidase remains to be established, the present results suggest certain chemical features that may be characteristic of the cofactor. Thus it seems that the carbonyl-bearing residue must contain a function or functions that can participate in stable cofactor–protein linkages. Moreover, the absorption maximum of the enzyme phenylhydrazone at 455 nm suggests that the cofactor possesses significant resonance capability, consistent with aromatic character of the carbonyl moiety. Since hydrazine prevents incorporation of phenylhydrazine, it is also likely that a stable hydrazone of the enzyme carbonyl is produced. Stable hydrazones are more typical products of the reaction of hydrazine with aromatic rather than non-aromatic carbonyl moieties. Accordingly, it is unlikely that a covalently bound pyruvoyl group, as found in microbial histidine decarboxylase (Huynh et al., 1984), is the carbonyl-bearing residue of lysyl oxidase. These considerations similarly argue against peptidyl α-aminoadipic δ-semialdehyde, the potential product of lysyl oxidase action on itself, as the site of carbonyl reactivity described in these studies. A complex o-quinone, 2,7,9-tricarboxy-1H-pyrrolo[2,3-f]-quinoline-4,5-dione, semi-systematically named pyrro-loquinoline quinone, has been identified as a cofactor in bovine plasma monoamine oxidase (Lobenstein-Verbeek et al., 1984). This compound is aromatic, exhibits carbonyl reactivity and redox behaviour and contains three carboxy groups as potential participants in amide formation with enzyme amino functions. Thus there is precedent for an enzyme cofactor with chemical characteristics similar to those expected of the carbonyl moiety in lysyl oxidase.

The present results thus contrast with earlier conclusions that PLP is a cofactor in chick aortic lysyl oxidase (Bird & Levene, 1982). Although it remains possible that lysyl oxidase of different tissues or species may contain different cofactors, other, more indirect, effects also may account for the physiological perturbations of cross-linkage profiles related to PLP contents. Thus cross-linkage profiles in lung elastin of vitamin B-6-deficient animals are altered to resemble the shift in cross-linkage contents resulting from treatment with D-penicillamine (Myers et al., 1985), and L-homocysteine, which is a substrate of

cystathionine β-synthase, a PLP-dependent enzyme, was increased in the serum of vitamin B-6-deficient animals (Myers et al., 1985). D-Penicillamine alters cross-linkage formation by trapping free aldehyde products of lysyl oxidase action as thiazolidine adducts (Nimni, 1968; Siegel, 1977). Since both homocysteine and penicillamine are aminothiols capable of forming thiazolidines with aldehydes, it is conceivable that excess homocysteine may mediate the change in cross-linkage content induced by vitamin B-6 deficiency by such a mechanism (Myers et al., 1985). Nevertheless it remains possible that lysyl oxidases of different species and different tissues contain different carbonyl cofactors, a possibility that might be related to the greater decrease in extractable activity of the cartilage enzyme than the aortic enzyme in vitamin B-6-deficient chicks (Murray et al., 1978).

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